

# Molecular Aspects of Polyene- and Sterol-Dependent Pore Formation in Thin Lipid Membranes

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**ABSTRACT** Amphotericin B modifies the permeability properties of thin lipid membranes formed from solutions containing sheep red cell phospholipids and cholesterol. At  $10^{-6}$  M amphotericin B, the DC membrane resistance fell from  $\approx 10^8$  to  $\approx 10^2$  ohm-cm<sup>2</sup>, and the membranes became Cl<sup>-</sup>-, rather than Na<sup>+</sup>-selective; the permeability coefficients for hydrophilic nonelectrolytes increased in inverse relationship to solute size, and the rate of water flow during osmosis increased 30-fold. These changes may be rationalized by assuming that the interaction of amphotericin B with membrane-bound sterol resulted in the formation of aqueous pores. *N*-acetylamphotericin B and the methyl ester of *N*-acetylamphotericin B, but not the smaller ring compounds, filipin, rimocidin, and PA-166, produced comparable permeability changes in identical membranes, and amphotericin B and its derivatives produced similar changes in the properties of membranes formed from phospholipid-free sterol solutions. However, amphotericin B did not affect ionic selectivity or water and nonelectrolyte permeability in membranes formed from solutions containing phospholipids and no added cholesterol, or when cholesterol was replaced by either cholesterol palmitate, dihydrotachysterol, epicholesterol, or  $\Delta^5$ -cholesten-3-one. Phospholipid-free sterol membranes exposed to amphotericin B or its derivatives were anion-selective, but the degree of Cl<sup>-</sup> selectivity varied among the compounds, and with the aqueous pH. The data are discussed with regard to, first, the nature of the polyene-sterol interactions which result in pore formation, and second, the functional groups on amphotericin B responsible for membrane anion selectivity.

## INTRODUCTION

Thin lipid membranes formed from sheep red cell lipid-decane solutions have a high electrical resistance, are permselective for Na<sup>+</sup> or K<sup>+</sup> instead of

Cl<sup>-</sup> (1), and are rather impermeable to small hydrophilic solutes (2). The structurally related polyene antibiotics, nystatin and amphotericin B, reduced to a striking degree the DC resistance of these membranes, which became anion-permselective in the low resistance state (3). Concomitantly, amphotericin B produced a 30-fold increment in the rate of water flux through the membranes during osmosis and graded increases in nonelectrolyte permeability that varied inversely with molecular size (2). Accordingly, we suggested that the interactions of amphotericin B with these membranes resulted in the formation of aqueous pores (2). Both the nystatin-dependent increases in ionic permeability (3) and the amphotericin B-dependent increases in nonelectrolyte permeability (2) were proportional to a high power (approximately 2.5–4.5) of the antibiotic concentration in the aqueous phases bathing the membranes, and absolutely dependent on the presence of cholesterol in the lipid solutions used to form membranes. Stated alternatively, it seemed likely that the primary unit responsible for the increases in water, electrolyte, and nonelectrolyte permeability was comprised of a multimolecular aggregate of polyene antibiotic with membrane-bound cholesterol (3). However, the experimental data provided no information concerning either the nature of the polyene-sterol interactions involved in the formation of such units, or whether such units modified membrane permeability directly or by cooperative interaction with membrane phospholipids. Furthermore, the molecular basis for polyene-dependent membrane anion selectivity was not defined.

This paper describes the effects of different polyene antibiotics on the permeability properties of thin lipid membranes formed from lipid solutions containing either various sterols and sheep red cell phospholipids or sterols but no phospholipids, and the effects of altering the pH of the aqueous phases on polyene-dependent membrane anion selectivity. The experimental data are compatible with the hypothesis that, in these membranes, one interaction required for the formation of aqueous pores is stereospecific hydrogen bonding between the hydrogens of 3  $\beta$ -OH groups of appropriate sterols and indeterminate sites on polyene antibiotics, such as amphotericin B, which have 36–38 rather than 28 ring atoms. Furthermore, under the appropriate conditions, polyene- and sterol-dependent pore formation may occur independently of membrane phospholipids. In addition, the data imply that amphotericin B-dependent membrane anion permselectivity may be referable primarily to the hydroxyl or carbonyl substituents on the antibiotic molecule, but that the amino and carboxyl moieties on the compound also contribute, to a lesser degree, to the regulation of membrane ionic selectivity.

Preliminary reports of some of these observations have appeared elsewhere (4, 5).

## METHODS

Optically black, thin lipid membranes separating two aqueous phases were formed by applying lipid solutions with a brush technique (6) to an aperture in a polyethylene diaphragm separating two aqueous phases. The lipid solutions used to form membranes (membrane solutions) were of two types. One type contained acetone-extracted, high potassium (HK) sheep red blood cell lipids (92% phospholipid [3]) dissolved in decane. Varying amounts of different sterols, indicated in the text, were added to these solutions and the total lipid concentration was in the range from 25 to 40 mg/ml. The second type of membrane solution was similar to those described by Tien et al. (7, 8). In these phospholipid-free solutions, equimolar amounts of cholesterol (Calbiochem, Los Angeles, Calif.) and 7-dehydrocholesterol (Calbiochem, Los Angeles, Calif.) were dissolved, at a final concentration of 13 mg/ml, in solutions containing decane:chloroform in a volume ratio of 8:3.

The composition of the aqueous phases is indicated in the text. Unless otherwise indicated (Tables VI and VII), the pH of the unbuffered aqueous phases was in the range from 5.8 to 6.0 (1-3).

Except for the osmotic water flux experiments described in Table IV, all other experiments were carried out in Lucite chambers identical to those described previously (2), except that the chambers were surrounded by water jackets. By perfusing the jackets with thermostatted water, it was possible to regulate the temperature of the aqueous phases bathing the membranes to  $\pm 0.5^\circ\text{C}$ , in the range from  $20^\circ$  to  $45^\circ\text{C}$ .

For some osmotic water flux experiments (Table IV), we used a closed water-jacketed rear chamber (volume  $\sim 1.5$  ml) similar to the one described previously (1). A fluid-filled "U" tube (2) which could be closed by a two-way stopcock connected the front and rear chambers. With the use of a simultaneous infusion-withdrawal pump (Model 1201; Harvard Instrument Co., Boston, Mass.) connected to the rear chamber, and with the U tube open to compensate for any inequalities of flow, the contents of the rear chamber could be exchanged at a rate of 0.8 ml/min for 10-20 min without rupturing the membranes. After perfusion, all connections to the rear chamber except the tubing connected to the micrometer used to measure net water flow (2) were sealed. The results with this type of chamber were indistinguishable from those observed in the other experiments. In particular, the net flux of water was related linearly to the osmotic pressure difference (in the range from 5 to 20 atmospheres) in the two aqueous phases bathing the membranes.

$P_{D_i}$  ( $\text{cm sec}^{-1}$ ), the tracer permeability coefficient for diffusion of water or the  $i$ th solute, was estimated from unidirectional tracer fluxes at zero volume flow. As indicated elsewhere (2), the minimum value of  $P_{D_i}$  which could be detected reproducibly under the experimental conditions was  $\sim 0.05 \times 10^{-4} \text{ cm sec}^{-1}$ . Accordingly, when the unidirectional tracer fluxes were negligibly small, the values of  $P_{D_i}$  were reported as less than  $0.05 \times 10^{-4} \text{ cm sec}^{-1}$ .  $P_f$  ( $\text{cm sec}^{-1}$ ), the osmotic water coefficient, was estimated from net water fluxes when the two aqueous phases bathing the membranes contained unequal concentrations of solute. The details of these experimental techniques have been presented elsewhere (2). As demonstrated previously (2), when

$P_{D_{H_2O}}$  was more than  $10^2$  times greater than  $P_{D_i}$  for a particular solute, the reflection coefficient (9) of that solute, estimated from net water flux experiments, was unity. Accordingly, in the present experiments,  $P_f$  was estimated when sucrose was the aqueous solute, since  $P_D$  sucrose was negligibly small with respect to  $P_{D_{H_2O}}$  either in the presence or absence of polyene antibiotics (Tables II, IV, V, VIII).

The electrical properties of the membranes, including DC resistance ( $R_m$ , ohm-cm<sup>2</sup>), zero frequency capacitance ( $C_m$ , μF/cm<sup>2</sup>), and membrane voltage ( $V_m$ , mv) were recorded as described previously (1, 3). The DC circuit was arranged so that the rear chamber was positive or negative and the front chamber was grounded.

An estimate of the relative ionic permeabilities of the membranes was made by computing ionic transference numbers ( $t_i$ , the transference number of the  $i$ th ion, is defined as the ratio of the membrane conductance of that ion to the total membrane conductance [1, 3]) from the steady-state membrane potentials ( $V_m$ ) when either the concentration of NaCl or the pH in the two aqueous phases bathing the membranes was different (1, 3, 10). When the aqueous phases contained NaCl and the contributions of H<sup>+</sup> and OH<sup>-</sup> to membrane conductance were considered, the expression for membrane potential was (1, 10):

$$V_m = t_{Na}E_{Na} + t_{Cl}E_{Cl} + t_H E_H + t_{OH}E_{OH}, \quad (1)$$

where  $E_i$  is the equilibrium potential of the  $i$ th ion (1). When the pH of both aqueous phases was identical, Equation 1 reduced to (10):

$$V_m^{Na} = t_{Na}E_{Na} + t_{Cl}E_{Cl}, \quad (2)$$

where  $V_m^{Na}$  is the membrane potential produced by unequal salt concentrations in the two aqueous phases. Similarly, when the salt concentration in both aqueous phases was identical, Equation 1 reduced to:

$$\frac{V_m^H}{E_H} = t_H + t_{OH}, \quad (3)$$

where  $V_m^H$  is the membrane potential produced when the pH of the two aqueous phases was different. Equation 3 has been stated incorrectly elsewhere (10). Furthermore, under either condition:

$$t_{Na} + t_{Cl} + t_H + t_{OH} = 1. \quad (4)$$

When it is assumed that the transference numbers are independent of concentration, simultaneous solution of Equations 1-4 yields

$$t_{Na} = \frac{V_m^{Na} + E_{Cl} \left( \frac{V_m^H}{E_H} - 1 \right)}{E_{Na} - E_{Cl}} \quad (5 a)$$

and

$$t_{Cl} = 1 - t_{Na} - \frac{V_m^H}{E_H} \quad (5 b)$$

In membranes formed from sheep red cell lipids dissolved in decane,  $(t_H + t_{OH})$  was less than one-tenth of  $(t_{Na} + t_{Cl})$ , when the pH of the aqueous phases was approximately 6.0 (10). In preliminary experiments, we observed that  $(t_H + t_{OH})$  was approximately zero when similar membranes were exposed to aqueous solutions (pH  $\sim$  6.0) containing the polyene drugs used in these studies. Accordingly, the ionic transference numbers listed in Tables I–V were computed from the values of  $V_m^{Na}$  and Equations 5 *a* and 5 *b*, assuming that  $V_m^H$  was zero. However, the contributions of  $H^+$  and  $OH^-$  to the membrane conductance in phospholipid-free membranes were substantial (Tables VI and VII). Consequently, in the experiments listed in Tables VI and VII, the ionic transference numbers were computed from Equations 3, 5 *a*, and 5 *b* and the experimental values for  $V_m^H$  and  $V_m^{Na}$ . Finally, it should be noted that the application of Equations 5 *a* and 5 *b* depends on certain assumptions, discussed in detail elsewhere (1, 3). There is reasonable experimental evidence (i.e.,  $V_m$  is related linearly to the logarithm of the activity of salt in the two aqueous phases bathing the membranes; cf. Equation 2) to indicate that these assumptions are valid for membranes formed from sheep red cell lipid solutions in the absence or presence of polyene antibiotics, if the activity ratio of salt in the two aqueous phases is  $\leq 50$  (1, 3). In preliminary experiments, qualitatively similar results were obtained on membranes formed from phospholipid-free solutions. Accordingly,  $V_m$  was measured, in all instances, when there was approximately a 10-fold difference in the salt or  $H^+$  concentration in the two aqueous phases bathing the membranes.

Amphotericin B (batch No. 91368-001) was kindly furnished by Miss Barbara Stearns (Squibb Institute for Medical Research, New Brunswick, N. J.). *N*-Acetyl-amphotericin B (batches No. VS-12-25-1 and MM-4735-1) and the methyl ester of *N*-acetyl-amphotericin B (batch No. MM 4735-47) were prepared and kindly furnished by Dr. Frank L. Weisenborn and his collaborators at the Squibb Institute for Medical Research. The methyl ester was prepared from *N*-acetyl-amphotericin B batch No. MM-4735-1. These workers assayed both samples of *N*-acetyl-amphotericin B with thin layer chromatography using an *n*-butanol–methanol–benzene–water–acetic acid solvent system (40:20:20:19:1, by volume) followed by bioautography of the chromatograms with both *Candida albicans* and *Candida tropicalis*. Sample VS-12-25-1 contained less than 0.2%, and sample MM-4735-1 less than 0.1%, of amphotericin B.<sup>1</sup> The methyl ester of *N*-acetyl-amphotericin B contained no titratable carboxyl groups, and produced a single spot on thin layer chromatography in a system containing *n*-butanol–methanol–benzene–water (2:1:1:1, by volume), pH 8.2.<sup>1</sup>

PA-166 (lot No. 5831-259-2) and rimocidin (lot No. 4157-48-4) were kindly supplied by Mr. Nathan Belcher and Dr. Sheldon Gilgore (Chas. Pfizer Co., Groton, Conn.). Epicholesterol was kindly furnished by Dr. Alan F. Hofman (Mayo Clinic, Rochester, Minn., and by Dr. R. S. Rosenfeld, Institute for Steroid Research, Montefiore Hospital, New York). Dihydrocholesterol, dihydrotachysterol, and  $\Delta^5$ -cholesten-3-one were purchased from Mann Research Laboratories, New York. The antibiotics were dissolved in either methanol or dimethylsulfoxide in concentrations sufficiently high that the final aqueous phase concentration of either of the solvents

<sup>1</sup> Weisenborn, F. L. Personal communication.

was less than 1% (2, 3). Other analytical determinations, techniques, and reagents were the same as in previous studies (1-3).

## RESULTS

### 1. Experiments with Sheep Red Cell Phospholipid Membranes

#### THE STEROL REQUIREMENT FOR AMPHOTERICIN B-DEPENDENT PORE FORMATION

In the concentration range,  $10^{-7}$ – $10^{-6}$  M, amphotericin B produced striking alterations in the transport properties of thin lipid membranes formed from solutions containing equimolar amounts of sheep red cell phospholipids and cholesterol (references 2, 3 and Table I). The changes in the permeability of the membranes to water and nonelectrolytes were rationalized by assuming

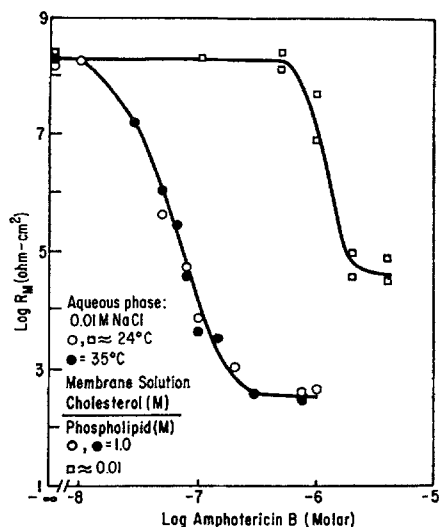


FIGURE 1. The relationship of the log DC membrane resistance ( $R_m$ , ordinate) to the log amphotericin B concentration in the aqueous phases (abscissa). The membrane solutions contained the indicated molar ratio of HK sheep red cell phospholipids and cholesterol dissolved in decane. The temperature and composition of the aqueous phases (pH  $\sim$  6.0) are indicated in the figure. Experimental details are given in Methods.

that the interaction of amphotericin B with membrane-bound cholesterol resulted in the formation of aqueous pores, whose effective radii were approximately 7–10.5Å (2).

The results of a series of experiments which were designed to evaluate the specificity of these interactions, with regard to the sterol, are illustrated in Figs. 1 and 2 and Tables I–III. Fig. 1 shows the effect of amphotericin B on the DC resistance of membranes formed from different lipid solutions. When these solutions contained equimolar amounts of cholesterol and phospholipid, the DC membrane resistance fell to  $\sim 3 \times 10^2$  ohm-cm<sup>2</sup> when the antibiotic concentration was approximately  $2\text{--}3 \times 10^{-7}$  M. In the concentration range, from  $10^{-8}$  to  $10^{-7}$  M, the slope of the relationship between the log membrane resistance and the log amphotericin B concentration was approximately

–5.5. These data are similar to those observed previously with nystatin (3, 11). However, in contrast to the results with nystatin (11) or amphotericin B derivatives (Fig. 3), the relationship between the log amphotericin B concentration and the log membrane resistance was independent of temperature, in the range from 24° to 35° C (Fig. 1).

TABLE I  
STEROL-DEPENDENT AND STEROL-INDEPENDENT  
EFFECTS OF AMPHOTERICIN B ON THE PERMEABILITY  
PROPERTIES OF THIN LIPID MEMBRANES

Membrane solution					
Added sterol	Sterol (M) P' lipid (M)	Amphotericin B	$P_{D_{H_2O}}$	$P_{D_{urea}}$	$P_f$
		M	$cm\ sec^{-1} \times 10^4$	$cm\ sec^{-1} \times 10^4$	$cm\ sec^{-1} \times 10^4$
None	~0.01	0	12.0±0.47 (5)	—	31.5±4.2 (9)
None	~0.01	4.0×10 <sup>-6</sup>	10.3 (2)	<0.05 (4)	33.4±5.5 (6)
Cholesterol	1.0	0	9.4±0.6 (6)	<0.05 (3)	22.9±4.6 (64)
Cholesterol	1.0	1.0×10 <sup>-6</sup>	16.3±0.5 (4)	8.6±1.5 (4)	549±143 (22)
			$R_m$	$V_m$	$t_{Na}$ $t_{Cl}$
			$ohm\ cm^2$	$mv$	
None	~0.01	0	1.7±0.8×10 <sup>8</sup> (5)	39.0±4.6 (5)	0.85   0.15
None	~0.01	4.0×10 <sup>-6</sup>	2.6±1.2×10 <sup>8</sup> (4)	38.2±3.3 (5)	0.84   0.16
Cholesterol	1.0	0	1.3±10 <sup>8</sup> (3)	41.0±4.1 (4)	0.86   0.14
Cholesterol	1.0	1.0×10 <sup>-6</sup>	4.1±1.7×10 <sup>8</sup> (5)	-41.2±3.5 (5)	0.14   0.86

The membrane solutions contained the indicated molar ratios of cholesterol and HK sheep red cell phospholipids in decane. The pH of the aqueous phases was ~6.0.  $P_f$  was measured at 23°C, and all other experiments were carried out at 35°C. When  $P_{D_{urea}}$  and  $P_{D_{H_2O}}$  were measured, the aqueous phases contained 0.01 M NaCl, 0.01 M urea; either urea-<sup>14</sup>C or THO was added to the rear chamber. The values of  $P_f$  in the absence of amphotericin B and at 1.0 × 10<sup>-6</sup> M amphotericin B were reported previously (2). For measurement of  $P_f$  at 4 × 10<sup>-6</sup> M amphotericin B, the membranes were formed in 0.01 M NaCl, 0.01 M sucrose; net water flux was measured when the front aqueous phase contained 0.01 M NaCl and higher concentrations of sucrose (in the range from 0.05 to 0.25 M).  $R_m$  was measured when the aqueous phases contained 0.01 M NaCl or 0.01 M NaCl, 0.01 M urea.  $V_m$  was recorded when the front chamber contained 0.1 M NaCl and the rear chamber contained 0.01 M NaCl. During the tracer flux experiments, both chambers were grounded; for the electrical measurements, only the front chamber was grounded. The results are expressed as the mean ± standard deviations (SD) for the number of observations listed in parentheses. Experimental details are given in the section on Methods.

In previous experiments,  $\leq 5 \times 10^{-7}$  M amphotericin B did not affect either the electrical resistance (3) or the water and nonelectrolyte permeability (2) of membranes formed from lipid solutions in which the molar ratio of cholesterol to phospholipid was  $\leq 0.05$ . However, Fig. 1 shows that when the membrane solutions contained no added cholesterol and the amphotericin B concentration exceeded 10<sup>-6</sup> M, the membrane resistance fell and ap-

proached a limiting value of approximately  $10^5$  ohm-cm<sup>2</sup> when the antibiotic concentration was in the range from  $2$  to  $4 \times 10^{-6}$  M. In this regard, it should be noted that, even after extraction with acetone (3), the molar ratio of cholesterol to phospholipid in sheep red blood cell lipids is approximately 0.01 (reference 3 and Fig. 1). Accordingly, it was relevant to exclude the possibility that the "sterol-independent" fall in membrane DC resistance was referable to an interaction of amphotericin B with limiting amounts of cholesterol instead of other membrane components.

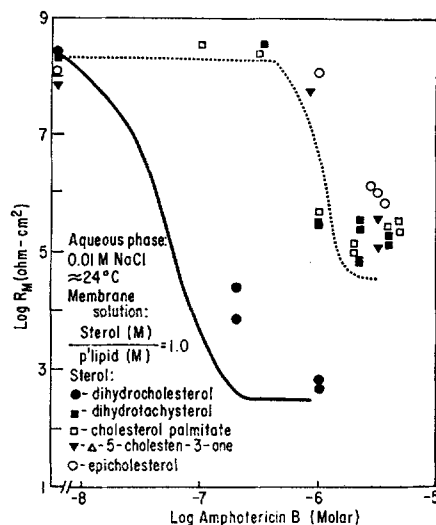


FIGURE 2. The effect of varying the composition of the membrane solutions on the amphotericin B-dependent fall in DC membrane resistance. The membrane solutions contained equimolar amounts of HK sheep red cell phospholipids and the indicated sterols dissolved in decane. The temperature and composition of the aqueous phase (pH  $\sim$  6.0) are indicated in the figure. The reference curves were redrawn from Fig. 1 (solid curve, equimolar amounts of cholesterol and phospholipid in the membrane solution; dotted curve, the molar ratio of cholesterol to phospholipid in the membrane solutions was  $\sim$ 0.01), and the axes have the same values as on Fig. 1. Experimental details are given in Methods.

Table I shows a comparison of the cholesterol-dependent and sterol-independent effects of amphotericin B on membrane permeability. When amphotericin B ( $10^{-6}$  M) was in the aqueous phases and the membrane solutions contained equimolar amounts of cholesterol and phospholipid, reduction in membrane resistance was accompanied by striking increases in water and urea permeability (2). Furthermore, such membranes were Cl<sup>-</sup>, rather than Na<sup>+</sup>-selective (3). In contrast, when the lipid solutions contained no added cholesterol, the fall in membrane resistance in the presence of  $4 \times 10^{-6}$  M amphotericin B was not associated with any significant change in either electrolyte, urea, or water permeability. Since these effects of ampho-



tericin B were both qualitatively and quantitatively different from those observed when cholesterol was added to the membrane solutions, it seems likely that at relatively high aqueous concentrations (i.e.,  $2-4 \times 10^{-6}$  M), amphotericin B may interact with other components of the membranes besides cholesterol.

TABLE II  
THE EFFECT OF AMPHOTERICIN B ON THE PERMEABILITY  
PROPERTIES OF DIHYDROCHOLESTEROL-PHOSPHOLIPID  
THIN LIPID MEMBRANES

Molecule	Amphotericin B		$P_{D_i}$	$P_f$
	M			
H <sub>2</sub> O	0		$9.3 \pm 1.0$ (9)	—
H <sub>2</sub> O	$10^{-6}$		$16.4 \pm 2.4$ (4)	—
Urea	0		$< 0.05$ (2)	—
Urea	$10^{-6}$		$10.3 \pm 1.0$ (4)	—
Glycerol	0		$< 0.05$ (2)	—
Glycerol	$10^{-6}$		2.7 (3)	—
Sucrose	0		$< 0.05$ (2)	$29.7 \pm 1.6$ (6)
Sucrose	$10^{-6}$		$\sim 0.1$ (3)	$539 \pm 31$ (5)

Aqueous phase		Ampho- tericin B	$R_m$	$V_m$	$t_{Na}$	$t_{Cl}$
Rear chamber	Front chamber					
M	M	M	$\text{ohm-cm}^2$	mo		
0.01 M NaCl	0.1 M NaCl	0	$1.4 \pm 0.6 \times 10^8$ (7)	$24 \pm 2.2$ (4)	0.70	0.30
0.01 M NaCl	0.1 M NaCl	$10^{-6}$	$2.8 \times 10^2$ (3)	$-36 \pm 3.2$ (5)	0.18	0.82

The membrane solutions contained equimolar amounts of dihydrocholesterol and HK sheep red cell phospholipids dissolved in decane, and all the aqueous phases were 35°C, pH  $\sim$  6.0. When  $P_{D_i}$  was measured, the aqueous phases contained 0.01 M NaCl and the indicated solute (0.01 M); the corresponding <sup>14</sup>C-tagged isotope was added to the rear aqueous phase. For  $P_{D_{H_2O}}$  determinations, the aqueous phases contained 0.01 M NaCl and 0.01 M urea; THO was added to the rear aqueous phase. For measurement of  $P_f$ , the membranes were formed in 0.01 M NaCl, 0.01 M sucrose; net water flux (rear chamber to front chamber) was measured when the front aqueous phase contained 0.01 M NaCl and at least two different, greater concentrations of sucrose (in the range from 0.05 to 0.5 M). When  $R_m$  was measured, both aqueous phases contained 0.01 M NaCl;  $V_m$  was recorded when the aqueous phases contained the salt concentrations indicated in the table. The results are expressed as in Table I, and experimental details are given in the section on Methods.

Fig. 2 shows the effects of amphotericin B on the dc resistance of thin lipid membranes formed from lipid solutions containing equimolar amounts of HK sheep red cell phospholipids and the different sterols indicated in the figure. The cholesterol-dependent and sterol-independent curves were redrawn from Fig. 1 and the points represent the experimental values. Under these conditions, effects similar to the cholesterol- and amphotericin B-dependent reduction in membrane resistance occurred only when the lipid

solutions contained dihydrocholesterol. In contrast, the DC resistances of membranes formed from lipid solutions containing the remaining sterols listed in Fig. 2 were in the range from  $10^6$  to  $10^8$  ohm-cm<sup>2</sup>, when the amphotericin B concentration was  $2-4 \times 10^{-6}$  M.

Table II illustrates the effects of amphotericin B on the water, electrolyte, and nonelectrolyte permeability of membranes formed from solutions containing equimolar amounts of dihydrocholesterol and HK sheep red cell phospholipids. In the absence of the antibiotic, the permeability properties of these membranes were similar to those of their cholesterol-containing counterparts (references 1, 2 and Table I). Furthermore, when amphotericin B ( $10^{-6}$  M) was present in the aqueous phases, the results were the same, within experimental error, as those observed in like membranes formed from solutions containing cholesterol, rather than dihydrocholesterol (references 2, 3 and Table I).

There are no a priori reasons for assuming that the sterols listed in Fig. 2, other than dihydrocholesterol, were not incorporated into the membranes. However, this possibility might account for the apparent inability of these sterols to modify the sterol-independent effects of amphotericin B on membrane resistance (Fig. 2). Finkelstein and Cass (13) have shown that the water permeability of thin lipid membranes formed from lecithin solutions decreased when the molar ratio of either ergosterol or cholesterol to phospholipid in the membrane solutions was greater than 0.8. Accordingly, it was of interest to evaluate the effects of amphotericin B on the permeability properties of membranes whose water permeability, in the absence of the antibiotic, had been reduced by the addition of relatively high concentrations of the sterols listed in Fig. 2 to the lipid solutions (Table III). In these experiments,  $P_{D_{H_2O}}$ , rather than  $P_f$ , was chosen as an index of water permeability, since the former assay, in this laboratory, is more convenient and slightly more reproducible than the determination of  $P_f$ . Although the values of  $P_{D_{H_2O}}$  may be modified by unstirred layers (2, 14-17), it seemed reasonable to attribute differences in the values of  $P_{D_{H_2O}}$ , when all experimental conditions except the composition of the membrane solutions were kept constant, to alterations in the composition of the membranes.

When amphotericin B was absent, it is evident that the addition of either  $\Delta^5$ -cholesten-3-one, dihydrotachysterol, or epicholesterol to the membrane solutions reduced significantly the value of  $P_{D_{H_2O}}$ , with reference to membranes formed from solutions containing no added sterols (Tables I and III). These sterols did not modify, to any appreciable degree, the other membrane properties listed in Table III, except in the case of epicholesterol, when the membrane resistance was reduced to  $2.9 \pm 1.2 \times 10^7$  ohm-cm<sup>2</sup>. In particular, a comparison of Tables I and III shows that the effects of  $4 \times 10^{-6}$  M amphotericin B on the permeability properties of membranes formed from

lipid solutions containing either these sterols or no added sterols were indistinguishable. Although the data are not shown in Table III, similar effects were observed when the membrane solutions contained cholesterol palmitate, and the molar ratio of sterol to phospholipid was 1.8. Thus, to the extent that a reduction in  $P_{D_{H_2O}}$  reflects the inclusion of a particular sterol into the membranes, these data indicate clearly that the amphotericin B-dependent reduction in membrane resistance which is accompanied, *pari passu*, by membrane anion selectivity and increases in water and nonelectrolyte permeability, depends on an interaction of this antibiotic with membrane-

TABLE III  
THE STEROL REQUIREMENT FOR AMPHOTERICIN B-DEPENDENT PORE FORMATION IN THIN LIPID MEMBRANES

Membrane solution		Amphotericin B	$P_{D_{H_2O}}$	$P_{D_{urea}}$	$R_m$	$V_m$	$t_{Na}$	$t_{Cl}$
Added sterol	$\frac{\text{Sterol (M)}}{\text{P' lipid (M)}}$							
		M	$cm \text{ sec}^{-1} \times 10^4$	$cm \text{ sec}^{-1} \times 10^4$	$ohm\text{-}cm^2$	$mv$		
$\Delta^5$ -Cholestene-3-one	2.5	0	$6.7 \pm 2.1$ (6)	<0.05 (3)	$1.2 \pm 0.4 \times 10^8$ (4)	$37.5$ (3)	0.83	0.17
$\Delta^5$ -Cholestene-3-one	2.5	$4.0 \times 10^{-6}$	$5.3 \pm 0.6$ (5)	<0.05 (3)	$2.9 \pm 1.4 \times 10^5$ (5)	$35.1$ (3)	0.81	0.19
Dihydrotachysterol	2.0	0	$7.7 \pm 0.9$ (5)	—	$1.1 \times 10^8$ (3)	$37.7 \pm 3.8$ (4)	0.83	0.17
Dihydrotachysterol	2.0	$4.0 \times 10^{-6}$	9.0 (2)	<0.05 (2)	$3.9 \pm 2.8 \times 10^4$ (6)	$39.7 \pm 4.8$ (4)	0.85	0.15
Epicholesterol	1.8	0	$8.2 \pm 0.8$ (2)	<0.05 (2)	$2.9 \pm 1.2 \times 10^7$ (4)	$38.0 \pm 4.1$ (4)	0.84	0.16
Epicholesterol	1.8	$4.0 \times 10^{-6}$	9.0 (2)	<0.05 (2)	$4.9 \pm 2.2 \times 10^5$ (4)	$38.6 \pm 1.4$ (6)	0.84	0.16

The membrane solutions contained HK sheep red cell phospholipids and the indicated sterols dissolved in decane; the molar ratio of sterol to phospholipid is indicated in the table. The aqueous phases were 35°C, pH ~ 6.0,  $V_m$  was measured when the rear chamber contained 0.01 M NaCl and the front chamber contained 0.1 M NaCl. The data are reported as in Table I. Experimental details are given in Table I and under Methods.

bound sterols having a 3  $\beta$ -OH group and an intact B ring (references 4, 5 and Fig. 5).

#### THE EFFECTS OF AMPHOTERICIN B DERIVATIVES ON MEMBRANE PERMEABILITY

Nystatin and amphotericin B contain, respectively 36–37 and 38 ring atoms (19, 20). Each of these compounds has a number of hydroxyl substituents and a carboxyl and an amino substituent (19). In order to assess the contributions of the amino and carboxyl groups to the interactions of amphotericin

B with membrane-bound cholesterol, we studied the effects of *N*-acetyl-amphotericin B and the methyl ester of *N*-acetyl-amphotericin B on the permeability properties of membranes formed from lipid solutions containing equimolar amounts of cholesterol and sheep red cell phospholipids.

Fig. 3 illustrates the effects of these compounds on the DC membrane resistance. At 23°C, both *N*-acetyl-amphotericin B ( $\sim 10^{-6}$  M) and the methyl ester of *N*-acetyl-amphotericin B ( $\sim 5 \times 10^{-6}$  M) reduced the electrical membrane resistance to less than  $10^3$  ohm-cm<sup>2</sup>. In this respect, the effects of these compounds were similar to those of amphotericin B. However, certain dis-

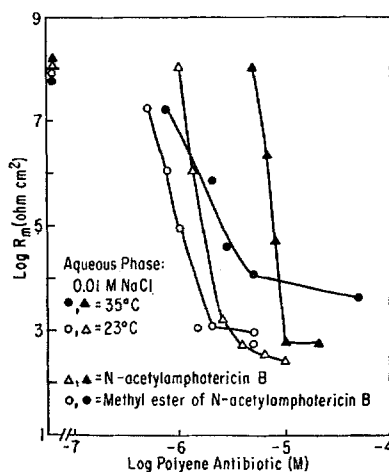


FIGURE 3. The effect of amphotericin B derivatives (abscissa) on the log DC membrane resistance ( $R_m$ , ordinate). The membrane solutions contained equimolar amounts of HK sheep red cell phospholipids and cholesterol dissolved in decane. The aqueous phases contained 0.01 M NaCl (pH  $\sim$  6.0) and the amphotericin B derivatives indicated in the figure. The temperature of the aqueous phases is shown in the figure. Experimental details are given in Methods.

crepancies were also evident. A comparison of Figs. 1 and 3 indicates that, at 23°C, the effective range of concentrations of these derivatives, with respect to a reduction in membrane resistance, was approximately 10 times greater than that of the parent compound, amphotericin B. Furthermore, in contrast to amphotericin B, the DC membrane resistance, at a particular concentration of *N*-acetyl-amphotericin B or the methyl ester of *N*-acetyl-amphotericin B (in the range from  $10^{-6}$  to  $10^{-5}$  M), was greater at 35°C than at 23°C. Finkelstein and Cass have observed a similar effect in the case of nystatin (11). The reasons for these differences are not clear, although they may depend, at least in part, on the equilibrium distribution of these agents between aqueous and membrane phases.

It was particularly relevant to exclude the possibility that the fall in mem-

brane resistance was due to contamination of these compounds with amphotericin B. The *N*-acetylamphotericin B (sample MM-4735-1) used both in these experiments and for the preparation of the methyl ester of *N*-acetylamphotericin B contained less than 0.1% amphotericin B. A comparison of Figs. 1 and 3 (at 23°C or 35°C) indicates that approximately 1% contamination would be required if the effects shown in Fig. 3 were due solely to an amphotericin B contaminant. Consequently, it is likely that these results were in fact dependent on the derivatives of amphotericin B. Additional support for this hypothesis will be provided below (Fig. 4 and Table VII).

Table IV indicates that, in these membranes, either *N*-acetylamphotericin

TABLE IV  
THE EFFECT OF AMPHOTERICIN B DERIVATIVES ON THE  
PERMEABILITY PROPERTIES OF CHOLESTEROL-PHOSPHOLIPID  
THIN LIPID MEMBRANES

Molecule	<i>N</i> -acetylamphotericin B ( $3 \times 10^{-6}M$ )		<i>N</i> -acetylamphotericin B, methyl ester ( $8.0 \times 10^{-6}M$ )	
	$PD_i$	$P_f$	$PD_i$	$P_f$
	$cm\ sec^{-1} \times 10^4$	$cm\ sec^{-1} \times 10^4$	$cm\ sec^{-1} \times 10^4$	$cm\ sec^{-1} \times 10^4$
H <sub>2</sub> O	15.50 (3) [14.3-16.1]	—	—	—
Urea	7.00 (3) [6.90-7.00]	—	7.13±0.81 (6)	—
Glycerol	2.60 (3) [2.30-2.90]	—	2.02 (3) [1.78-2.40]	—
Sucrose	~0.08 (3) [~0.05-0.12]	528±20 (4)	~0.10 (3) [~0.06-0.14]	298±62 (4)

Aqueous phase		<i>N</i> -acetylamphotericin B ( $10^{-6}M$ )			<i>N</i> -acetylamphotericin B, methyl ester ( $2.5 \times 10^{-6}M$ )		
Rear chamber	Front chamber	$V_m$	$t_{Na}$	$t_{Cl}$	$V_m$	$t_{Na}$	$t_{Cl}$
$M$	$M$						
0.01 NaCl	0.10 NaCl	-32.2±0.8 (4)	0.22	0.78	-46.8±1.1 (5)	0.07	0.93

The membrane solutions contained equimolar amounts of HK sheep red cell phospholipids and cholesterol in decane. The pH of the aqueous phases was ~6.0. The temperature was 35°C for the experiments with *N*-acetylamphotericin B and 23°C for the experiments with the methyl ester of *N*-acetylamphotericin B. The experimental values are reported either as the mean ± SD or the mean and range of values, indicated in brackets, for the number of observations listed in parentheses. Control data for these experiments (i.e., no polyene antibiotic) are given in Table I, under Results, and in references 1-3. Experimental details are given in the section on Methods and Table I.

B or the methyl ester of *N*-acetylamphotericin B produced both striking increases in  $P_f$  and graded increases in nonelectrolyte permeability, which varied inversely with solute size. Under these conditions, the values of  $P_{D_{\text{urea}}}$  and  $P_{D_{\text{glycerol}}}$  (Table IV) were approximately 30% lower than those observed in identical membranes at  $10^{-6}$  M amphotericin B (reference 2 and Table I). Furthermore, the increment in  $P_f$  due to the methyl ester of *N*-acetylamphotericin B was less than that observed with *N*-acetylamphotericin B (Table IV) or amphotericin B (reference 2 and Table I). A comparison of Tables I and IV indicates that, in identical membranes, the values of  $t_{Cl}$  were 0.93, 0.86, and 0.78 for, respectively,  $2.5 \times 10^{-6}$  M methyl ester of *N*-acetylamphotericin B,  $10^{-6}$  M amphotericin B, and  $10^{-5}$  M *N*-acetylamphotericin B. Although these differences are relatively small, these data are in accord with the observations on sterol-free membranes (Table VII).

#### THE EFFECT OF POLYENE ANTIBIOTICS HAVING FEWER RING ATOMS ON MEMBRANE PERMEABILITY

The pentaene antibiotic, filipin, is a lactone having 28 ring atoms and 9 hydroxyl groups, but no amino or carboxyl substituents (21). In membranes formed from lipid solutions containing equimolar amounts of cholesterol and phospholipid, this compound produced striking membrane instability (defined in Table V), but did not modify significantly either the total electrical membrane conductance or individual ionic conductances (3). Rimocidin is a tetraene antibiotic having a 28-membered lactone ring, 8 hydroxyl substituents, an amino substituent but no carboxyl group (22), while the tetraene antibiotic PA-166 contains both amino and carboxyl substituents (19). The effects of these two compounds on the permeability properties of these membranes are shown in Table V. In each instance, it is clear that concentrations of these antibiotics only slightly less than those required to produce membrane instability did not alter significantly DC membrane resistance, ionic selectivity, or urea or water permeability.

#### 2. Studies on Phospholipid-Free Thin Lipid Membranes

##### FORMATION OF PHOSPHOLIPID-FREE MEMBRANES

Recently, Tien et al. described the formation of thin lipid membranes from phospholipid-free hydrocarbon solutions containing either oxidized cholesterol or 7-dehydrocholesterol (7). The thickness of these membranes, estimated by optical methods, was approximately 40 Å (7). Subsequently, these workers (8) and others (23, 24) formed membranes from saturated solutions of recrystallized cholesterol in dodecane, but only when the aqueous phases contained the cationic surfactant hexadecyltrimethylammonium bromide. Furthermore, Tien and Diana noted that if the salt concentration in the

aqueous phases exceeded 0.01–0.1 M, these membranes were unstable and had DC resistances which varied widely (25).

In this laboratory, thin lipid membranes could be formed from saturated solutions of oxidized cholesterol in decane, but not from a number of lipid solutions containing either 7-dehydrocholesterol or cholesterol, when the aqueous phases contained 0.01–0.1 M NaCl, pH ~6.0, and the temperature was in the range from 25° to 38°C. However, membranes were generated reproducibly at 38°C. from solutions containing equimolar amounts of cholesterol and 7-dehydrocholesterol dissolved in decane:chloroform (8:3, by

TABLE V  
THE EFFECT OF SMALLER RING POLYENE ANTIBIOTICS ON  
THE PERMEABILITY PROPERTIES OF THIN LIPID MEMBRANES

Antibiotic	Concentration	$R_m$	$V_m$	$t_{Na}$	$t_{Cl}$	$P_{D_{urea}}$	$P_f$
		<i>ohm-cm<sup>2</sup></i>				<i>cm sec<sup>-1</sup> × 10<sup>4</sup></i>	<i>cm sec<sup>-1</sup> × 10<sup>4</sup></i>
Rimocidin	$3-6 \times 10^{-5}$	$4.1 \pm 1.4 \times 10^7$ (10)	$38.2 \pm 4.2$ (8)	0.84	0.16	<0.05 (9)	$29.5 \pm 2.8$ (6)
Rimocidin	$\geq 10^{-4}$	Membrane instability					
PA-166	$2-4 \times 10^{-6}$	$1.6 \pm 1.3 \times 10^8$ (7)	$45.0 \pm 3$ (4)	0.9	0.1	<0.05 (6)	$30.8 \pm 1.9$ (5)
PA-166	$\geq 10^{-5}$	Membrane instability					

The membrane solutions contained equimolar amounts of HK sheep red cell phospholipids and cholesterol dissolved in decane, and all aqueous phases were ~23°C, pH ~6.0. The concentrations of polyene drugs used in these experiments are listed in the table. The data are reported as in Table I. The term "membrane instability" indicates that, at those concentrations of polyene antibiotic, the membranes which were formed (at least ten in each instance) solidified within 5 min of formation, and ruptured by tearing in approximately 10 min, as was observed previously with filipin (6). Control data for these experiments (i.e., no polyene antibiotic) are given in Fig. 1, Table I, under Results, and in references 1–3. Experimental details are given in Table I and under Methods.

volume). Under these conditions, the composition and pH of the aqueous solutions could be varied (Tables VI and VIII), the DC membrane resistances were reproducible (Table VI), and an aqueous surfactant was not required. The lipids which were applied to the membrane aperture drained very rapidly, and interference colors were noted within 20 sec. Most commonly, the membranes became optically black within 2 min and were stable for 30–45 min, but their duration rarely exceeded 60 min. It should be noted that membranes could not be formed reproducibly if the volume ratio of decane:chloroform in the lipid solvent were greater than eight, or at temperatures below approximately 32°C. In either of these circumstances, particulate material appeared in the membranes, which ruptured in 5–10 min.

## ELECTRICAL PROPERTIES

The salient electrical properties of these membranes are shown in Table VI. The DC membrane resistance was  $1.30 \pm 0.71 \times 10^6$  ohm-cm<sup>2</sup>, or approximately  $10^1$ – $10^2$  times less than the electrical resistance of membranes formed under comparable conditions from solutions of sheep red cell lipids in decane

TABLE VI  
ELECTRICAL PROPERTIES OF  
PHOSPHOLIPID-FREE THIN LIPID MEMBRANES

Aqueous phase 0.01 M NaCl, pH ~ 6.0				$R_m$ ohm-cm <sup>2</sup> $1.30 \pm 0.71 \times 10^6$ (9)	$C_m$ $\mu F$ cm <sup>-2</sup> $0.52 \pm 0.05$ (6)		
Aqueous phase				$V_m$	$t_{Na}$	$t_{Cl}$	$t_{(H+OH)}$
Rear chamber		Front chamber					
NaCl	pH	NaCl	pH				
M		M		mv			
0.10	2.4	0.01	2.4	~0	~0	~0	~1.0
0.10	2.4	0.10	3.4	$-61.0 \pm 2.7$ (4)			
0.10	6.0	0.01	6.0	$-10.2 \pm 2.2$ (5)	0.24	0.07	0.69
0.10	6.0	0.10	5.0	$42.6 \pm 2.1$ (5)			
0.10	10.55	0.01	10.55	-12 (3)	0.21	0.01	0.78
0.10	10.55	0.10	11.30	-35 (3)			

The composition of the phospholipid-free membrane solutions is indicated in the Methods, and the temperature of the aqueous phases was uniformly 38°C. The pH of the unbuffered aqueous phases was ~6.0, and was varied by the addition of NaOH or HCl. At pH 10.55, the NaOH which was added altered negligibly the Na<sup>+</sup> concentration of the aqueous phases. Consequently, when the front chamber contained 0.01 M NaCl,  $E_{Na} \cong -57.4$  mv. However, at pH 2.4, the HCl added to the aqueous phases altered significantly the Cl<sup>-</sup> concentration. At that pH, when the front chamber contained 0.01 M NaCl,  $E_{Na} = -57.6$  mv and  $E_{Cl} = 49$  mv. The DC membrane resistance ( $R_m$ ), zero frequency capacitance ( $C_m$ ) and membrane potential ( $V_m$ ) were measured as described in Methods, and are expressed as the mean  $\pm$  SD for the number of observations shown in parentheses. The ionic transference numbers were computed from the mean values of  $V_m$  and Equations 3, 5 a, and 5 b, as described in the Methods.

(references 1, 3 and Fig. 1), lecithin in decane (26), oxidized cholesterol in octane, or cholesterol-dodecyl acid phosphate (27). In contrast, the electrical resistance of lecithin:tetradecane membranes, when the lipid solvent contained chloroform-methanol, was also in the range of  $10^6$  ohm-cm<sup>2</sup> (28, 29). The zero frequency capacitance of these membranes was  $0.52 \pm 0.05 \mu F$  cm<sup>-2</sup> (Table VI) in accord with the observations on membranes formed from solutions containing oxidized cholesterol and octane (27) or lecithin:cholesterol (molar ratio 1:2) in decane (30).



A remarkable property of these membranes was their high degree of selectivity for protons and hydroxyl anions (Table V). At pH 6.0,  $t_{(\text{H} + \text{OH})}$  was approximately 3 and 10 times greater, respectively, than  $t_{\text{Na}}$  and  $t_{\text{Cl}}$ , when the concentration of NaCl was, at a minimum,  $10^3$  times greater than the  $\text{H}^+$  concentration. Although  $t_{\text{H}}$  and  $t_{\text{OH}}$  could not be measured directly, it is reasonable to assume that  $t_{(\text{H} + \text{OH})}$  was, to a close approximation,  $t_{\text{H}}$  at pH 2.5 and  $t_{\text{OH}}$  at pH 10.55. Accordingly, the ionic transference numbers in Table VI imply that the electrical conductance of these membranes may have been due almost exclusively to protons, at pH 2.5, and in large part to hydroxyl anions at pH 10.55. In contrast,  $t_{(\text{H} + \text{OH})}$  did not exceed 0.1, in the pH range from 3.5 to 5.8, in high resistance membranes formed from sheep red cell lipid-decane solutions (10).

In order to evaluate the possibility that the relatively low DC resistance and high  $\text{H}^+$  and  $\text{OH}^-$  permeability of the phospholipid-free membranes were due solely to the chloroform in the membrane solutions, thin lipid membranes were formed in 0.01 NaCl (pH  $\sim$ 6.0,  $38^\circ\text{C}$ ) from lipid solutions containing equimolar amounts of HK sheep red blood cell phospholipids and cholesterol dissolved in decane:chloroform (8:3, by volume). The DC resistance of three such membranes had a mean value of  $4.1 \times 10^7$  ohm-cm<sup>2</sup>,  $t_{\text{Na}}$  and  $t_{\text{Cl}}$  were respectively, 0.73 and 0.19, and  $(t_{\text{H}} + t_{\text{OH}})$ , in the pH range from 4.9 to 6.0, was 0.08. Accordingly, it is likely that the unusual  $\text{H}^+$  and  $\text{OH}^-$  conductances of the phospholipid-free membranes were referable to the membrane-bound sterols, although the mechanisms by which these ions traverse such membranes are not understood.

#### THE EFFECTS OF POLYENE ANTIBIOTICS ON ELECTRICAL PROPERTIES

The effects of amphotericin B, *N*-acetylamphotericin B, and the methyl ester of *N*-acetylamphotericin B on the electrical resistance of the phospholipid-free membranes are shown in Fig. 4. The DC resistance of these membranes fell when the amphotericin B concentration was greater than  $10^{-8}$  M, or when the concentration of the methyl ester of *N*-acetylamphotericin B exceeded  $5 \times 10^{-6}$  M. When the concentration of either of these two compounds was  $10^{-7}$  M, the membrane resistance was less than  $10^3$  ohm-cm<sup>2</sup>. Furthermore, *N*-acetylamphotericin B, in the concentration range from  $10^{-7}$  to  $10^{-6}$  M, produced comparable changes in the electrical resistance of these membranes. Thus, the concentrations of antibiotic required to reduce the electrical resistance of these membranes (Fig. 4), in comparison to sheep red cell phospholipid-cholesterol membranes (Figs. 1 and 3), were at least 10 times lower in the case of *N*-acetylamphotericin B and the methyl ester of *N*-acetylamphotericin B, but approximately the same for amphotericin B. In other words, *N*-acetylamphotericin B decreased the resistance of the phospholipid-free membranes at concentrations which were  $10^2$  times less than

those which would be required if the effects were due solely to the amphotericin B contaminant (<0.1%; cf. Methods).

Table VII illustrates the effects of these three compounds on the ionic permeability properties of the phospholipid-free membranes, when the pH varied from 2.5 to 10.5. With reference to Table VI, Table VII shows that, in the presence of either amphotericin B, *N*-acetylamphotericin B, or the methyl ester of *N*-acetylamphotericin B, these low resistance membranes (Fig. 4) were primarily Cl<sup>-</sup>-selective, and that  $t_{(H + OH)}$  was reduced three- to fourfold at pH 10.55 and to zero in the pH range from 2.5 to 6.0. In particular, it is noteworthy that at pH 6.0, the values of  $t_{Cl}$  were 0.95, 0.82, and 0.71 for, respectively, the methyl ester of *N*-acetylamphotericin B, ampho-

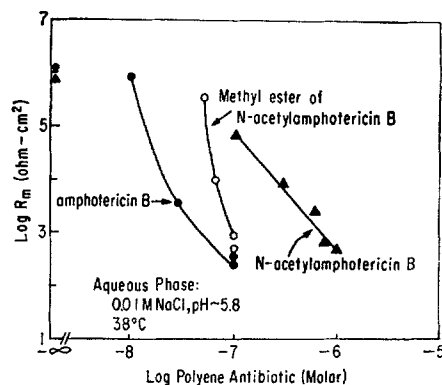


FIGURE 4. The effect of amphotericin B and its derivatives on the log DC membrane resistance ( $R_m$ , ordinate) of phospholipid-free membranes. The membranes were formed from phospholipid-free solutions, and the composition and temperature of the aqueous phases are indicated in the figure. Experimental details are given in Methods.

tericin B, and *N*-acetylamphotericin B, in close agreement with the effects produced by these compounds in the sheep red cell lipid-cholesterol membranes (Tables I and IV).

Table VII also shows a comparison of the effects of the three agents on the ionic transference numbers when the pH was varied. When the pH was reduced from  $\sim 6.0$  to 2.4, the values of  $t_{Cl}$  increased for amphotericin B and *N*-acetylamphotericin B to, respectively, 0.94 and 0.80, and the values of  $t_{Na}$  were correspondingly reduced. In contrast,  $t_{Na}$  and  $t_{Cl}$  were approximately the same at pH 2.4 and 6.0 in the presence of the methyl ester of *N*-acetylamphotericin B. When the pH was raised from 6.0 to 10.55, the value of  $t_{Cl}$  fell in the presence of each of the three agents, but the membranes remained anion-selective, since  $t_{(H + OH)}$  increased concomitantly. In this instance,  $t_{Na}$  remained the same for *N*-acetylamphotericin B, or increased appreciably less than the corresponding reduction in  $t_{Cl}$ , for amphotericin B or the methyl ester of *N*-acetylamphotericin B. Finkelstein and Cass (11)

noted that the nystatin-dependent  $\text{Cl}^-$  selectivity of thin lipid membranes formed from mixed brain phospholipids was pH-independent, in the range from 2.7 to 9.5.

TABLE VII  
THE EFFECT OF pH AND POLYENE ANTIBIOTICS ON THE  
IONIC PERMEABILITY PROPERTIES OF PHOSPHOLIPID-FREE  
THIN LIPID MEMBRANES

Aqueous phase				$V_m$	$t_{\text{Na}}$	$t_{\text{Cl}}$	$t_{(\text{H}+\text{OH})}$
Rear chamber		Front chamber					
NaCl	pH	NaCl	pH				
<i>M</i>		<i>M</i>		<i>mv</i>			
				<i>Amphotericin B = 10<sup>-7</sup> M</i>			
0.10	2.4	0.01	2.4	42.6±0.06 (5)	0.06	0.94	0
0.10	2.4	0.10	3.4	~0 (4)			
0.10	6.0	0.01	6.0	36.4±0.8 (4)	0.18	0.82	0
0.10	6.0	0.10	5.0	0 (2)			
0.10	10.55	0.01	10.55	21.4±1.2 (7)	0.26	0.62	0.12
0.10	10.55	0.10	11.30	-5.5±1.0 (5)			
				<i>N-acetylamphotericin B = 8 × 10<sup>-7</sup> M</i>			
0.10	2.4	0.01	2.4	28.0±0.7 (4)	0.20	0.80	0
0.10	2.4	0.10	3.4	0 (2)			
0.10	6.0	0.01	6.0	24.5±1.4 (6)	0.29	0.71	0
0.10	6.0	0.10	5.0	0 (2)			
0.10	10.55	0.01	10.55	14.0±1.4 (5)	0.29	0.58	0.18
0.10	10.55	0.10	11.30	-8.3±0.4 (5)			
				<i>Methyl ester of N-acetylamphotericin B = 10<sup>-7</sup> M</i>			
0.10	2.4	0.01	2.4	42.1±0.6 (4)	0.06	0.94	0
0.10	2.4	0.10	3.4	0 (1)			
0.10	5.9	0.01	5.9	50.7±0.7 (5)	0.05	0.95	0
0.10	5.0	0.10	5.9	0 (1)			
0.10	10.55	0.01	10.55	27.3±0.8 (4)	0.14	0.62	0.24
0.10	10.55	0.10	11.30	-11.0±0.6 (4)			

The aqueous phases contained the indicated concentrations of polyene antibiotics. Otherwise, the experiments were identical to those given in Table V.

THE EFFECT OF AMPHOTERICIN B ON WATER AND NONELECTROLYTE  
PERMEABILITY

Table VIII summarizes the effects of amphotericin B on the water and nonelectrolyte permeability properties of the phospholipid-free membranes. In the absence of amphotericin B, the nonelectrolyte permeability coefficients

were less than  $0.05 \times 10^{-4}$  cm sec<sup>-1</sup>, in accord with observations on membranes formed from a number of different lipid solutions (Tables I–III and references 2, 23, 24), and  $P_{D_{H_2O}}$  and  $P_f$  were, respectively,  $6.3 \pm 0.6 \times 10^{-4}$  cm sec<sup>-1</sup> and  $9.8 \pm 1.7 \times 10^{-4}$  cm sec<sup>-1</sup>. These water permeability data are similar to those observed in membranes formed either from phospholipid solutions containing relatively high mole fractions of sterols (reference 16 and Table III) or from various phospholipid-free sterol solutions (31). In view of the limited permeability of these membranes to nonelectro-

TABLE VIII  
THE EFFECT OF AMPHOTERICIN B ON  
THE WATER AND NONELECTROLYTE PERMEABILITY OF  
PHOSPHOLIPID-FREE THIN LIPID MEMBRANES

Molecule	Amphotericin B	$P_{D_i}$	$P_f$
	<i>M</i>	cm sec <sup>-1</sup> × 10 <sup>4</sup>	cm sec <sup>-1</sup> × 10 <sup>-4</sup>
H <sub>2</sub> O	0	6.3±0.6 (6)	—
H <sub>2</sub> O	1×10 <sup>-7</sup>	13.1 (3)	—
Urea	0	<0.05 (5)	9.8±1.7 (5)
Urea	1.2×10 <sup>-7</sup>	9.0±1.4 (4)	—
Glycerol	0	<0.05 (3)	—
Glycerol	1.2×10 <sup>-7</sup>	2.2±0.4 (4)	—
Ribose	0	<0.05 (3)	—
Ribose	1.2×10 <sup>-7</sup>	0.67±0.16 (5)	—
Sucrose	0	<0.05 (2)	—
Sucrose	1.2×10 <sup>-7</sup>	—	200±25 (7)

The composition of the phospholipid-free membrane solutions is indicated in the Methods. In all instances, the temperature of the aqueous phases was 38°C and the pH was approximately 6.0.  $P_{D_i}$  was measured as described in the Methods and Table I.  $P_f$  was measured as described in the Methods and Table I except that when amphotericin B was absent, urea rather than sucrose was used to produce an osmotic pressure difference in the two aqueous phases bathing the membranes. The data are expressed as in Table I.

lytes, it is reasonable to assume that the difference between  $P_{D_{H_2O}}$  and  $P_f$  was referable to unstirred layers in the aqueous phases adjacent to the membranes (2, 14–17) rather than to cooperative (i.e., laminar or quasilaminar) water flow during osmosis. In this instance, assuming that  $P_f$  approximated the true permeability coefficient for water diffusion (2, 16, 17), the effective thickness of the unstirred layers, computed as described elsewhere (2, 14–16), was approximately 130  $\mu$ , in reasonable agreement with previous estimates under similar experimental conditions (2).

When amphotericin B ( $1$ – $1.2 \times 10^{-7}$  M) was added to the aqueous phases, the nonelectrolyte permeability coefficients increased in inverse relationship

to solute size, and  $P_{D_{H_2O}}$  and  $P_f$  rose, respectively, approximately 2- and 20-fold. Furthermore, the solute permeability coefficients were in the same range, while the values of  $P_f$  were less than half of the comparable values in sheep red cell phospholipid membranes containing either cholesterol (reference 2 and Table I) or dihydrocholesterol (Table II). An understanding of whether these discrepancies in water permeability reflect subtle differences in the arrangement of polyene-sterol units evidently requires additional analysis.

However, it should be noted that  $1.2 \times 10^{-7}$  M amphotericin B (Table VIII) did not modify significantly either the appearance or duration of the membranes. In contrast, at  $\geq 1.7 \times 10^{-7}$  M amphotericin B, the membranes often solidified and ruptured within 10 min. Consequently, the net water flux or tracer flux experiments were not done at higher concentrations of the antibiotic. Lippe has observed that  $10^{-6}$  M amphotericin B did not affect the stability of membranes formed from cholesterol-dodecane solutions, when the aqueous phases contained hexadecyltrimethylammonium bromide (23). In such membranes,  $P_{D_{thiourea}}$  at  $10^{-6}$  M amphotericin B, was in the range of  $0.63 \times 10^{-4}$  cm sec<sup>-1</sup> (23), i.e., substantially lower than the value observed for  $P_{D_{urea}}$  in the present experiments at  $1.2 \times 10^{-7}$  M amphotericin B (Table VIII).

#### DISCUSSION

The observations presented above and elsewhere (2, 3, 11, 12, 23) indicate that polyene antibiotics may interact in a number of ways with thin lipid membranes separating two aqueous phases. For operational purposes, the cholesterol-dependent effects of amphotericin B on the dissipative transport properties of thin lipid membranes containing sheep red blood cell lipids (references 2, 3 and Fig. 1 and Table I) may be rationalized in terms of aqueous pores having effective radii of approximately 7–10.5 Å (2). Furthermore, it has been suggested that a multimolecular aggregate of polyene antibiotic and membrane-bound cholesterol is primarily involved in pore formation (2).

The present experiments (Figs. 1 and 2 and Tables I–III) show clearly that amphotericin B produced similar alterations in the permeability properties of thin lipid membranes when the lipid solutions contained sheep red blood cell phospholipids and either cholesterol or dihydrocholesterol, but not when the cholesterol in the lipid solutions and, by inference, in the membranes, was replaced by either cholesterol palmitate,  $\Delta^5$ -cholesten-3-one, epicholesterol, or dihydrotachysterol. Similarly, at appropriate concentrations, the effects of amphotericin B, *N*-acetylamphotericin B, and the methyl ester of *N*-acetylamphotericin B on the permeability properties of membranes formed either from solutions containing sheep red cell phospholipids and cholesterol (reference 2, Figs. 1 and 3, and Tables I and IV) or from phos-

pholipid-free sterol solutions (reference 2, Figs. 1 and 4, and Tables I, VII, and VIII) were comparable and in many instances quantitatively alike. Taken together, these observations suggest the hypothesis that, in these membranes, pore formation occurs independently of membrane-bound phospholipids and requires, at a minimum, hydrogen bonding between the equatorial 3-OH groups of appropriate sterols (cf. below) and sites on molecules such as amphotericin B.

The fact that  $\Delta^5$ -cholesten-3-one, the 3-ketone derivative of cholesterol, could not substitute for cholesterol in these interactions (Fig. 2 and Table III) implies that the hydrogens of the sterol 3-OH groups may bond directly to sites on the polyene molecule. The latter are indeterminate at present. However, in view of the similarities between the effects of amphotericin B, *N*-acetylamphotericin B, and the methyl ester of *N*-acetylamphotericin B, it seems unlikely that either the amino nitrogen or the oxygen on the carboxyl hydroxyl group of amphotericin B is the electronegative member of such hydrogen bonds. Conceivably, the carbonyl or free hydroxyl groups of amphotericin B and its derivatives, or nystatin (3, 11) could participate in these interactions. According to this view, the polyene-sterol unit involved in pore formation could be multimolecular (3), since a number of sterol molecules might bind to a polyene molecule, or alternatively, a number of polyene molecules might interact with each other. However, the experimental data are not adequate for distinguishing between these possibilities, or for evaluating the stoichiometry of such polyene-sterol units.

If one assumes that pore formation involves hydrogen bonding between sterol and polyene antibiotic, certain additional inferences are possible. Fig. 5 shows the structure of dihydrocholesterol in perspective formula (32). The cyclohexane rings, A, B, and C, are fixed firmly in the rigid and thermodynamically preferred "chair" configuration (33) by the *trans* fusion of rings A/B, B/C, and C/D (32). Introduction of a double bond between carbons 5 and 6, as in cholesterol, slightly modifies the form of the B ring, but does not alter substantially either the general conformation or the stability of the molecule (34, 35). A Corey-Pauling space-filling model of cholesterol had

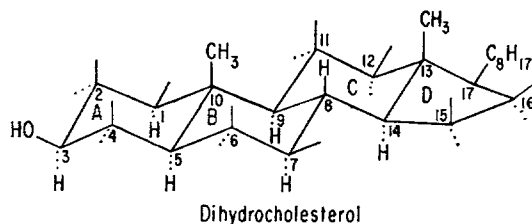


FIGURE 5. A perspective formula for dihydrocholesterol (32). The axial and equatorial substituents are indicated, respectively, by dotted and solid lines.

an inflexible rod-like shape and was contained in a circular cylinder whose approximate dimensions were: length, 19 Å; radius, 4 Å. A model of dihydro-tachysterol, which is identical to cholesterol except for a double bond between carbons 7 and 8 and no bond between carbons 9 and 10, had similar dimensions but considerably greater rotational mobility between the A and C rings in the long axis of the molecule. A second conformational aspect of interest relates to the 3-OH group. The orientation of this substituent is  $\beta$ , or equatorial (i.e., in the general plane of a cyclohexane ring [33]) in cholesterol and dihydrocholesterol, and axial (i.e., at right angles to the plane of a cyclohexane ring) in epicholesterol. It is noteworthy that a 3-OH group in the axial position is both less stable, because of steric repulsion by axial hydrogens on carbons 1 and 5 (Fig. 5), and, under certain conditions, e.g. acylation or precipitation by digitonin, less reactive than the equatorial form (36). These observations are consistent with the view that, in these membranes, the orientation of the functional 3-OH group of a sterol in a configuration favorable for the formation of pores depends, at least in part, on the equatorial position of this group and on the stability and rigidity of the perhydrocyclopentanophenanthrene skeleton.

Demel et al. showed that filipin interacted to a considerably greater extent with single lipid monomolecular layers containing cholesterol or cetyl alcohol instead of cholesterol acetate, and that these interactions were inhibited by the addition of 5 M urea to the aqueous phases (18). On the basis of these observations, these workers suggested that hydrogen bond formation was required for polyene-sterol interactions (18). However, it is evident that smaller ring polyene antibiotics such as filipin (3), rimocidin, or PA-166 (Table V) did not alter significantly the permeability properties of thin lipid membranes formed from lipid solutions containing cholesterol, even at concentrations slightly less than those required for membrane disruption. Accordingly, it seems likely that, in these membranes, the conformation of the polyene-sterol units which form pores is relatively specific and depends, among other factors, on the number of ring atoms,  $\approx 36$ –38 instead of 28, in the polyene molecule.

Under certain conditions, the amphotericin B-dependent membrane anion selectivity could be referred to sites other than the amino or carboxyl substituents, since membranes exposed to solutions containing either *N*-acetyl-amphotericin B or the methyl ester of *N*-acetyl-amphotericin B were also selective for  $\text{Cl}^-$ , rather than  $\text{Na}^+$  (Tables IV and VII). Accordingly, it is reasonable to infer that this phenomenon could be attributed to other functional groups, e.g. hydroxyl (11) or carbonyl, on amphotericin B. However, it is not evident from the available information about the structure of amphotericin B (19, 20) how such groups could acquire the relatively positive charge distribution one would predict on the basis of fixed charge theory (37),

although it is possible, for example, that such a charge distribution is due to electron withdrawal by other sites on the molecule. The experimental data (Tables I and III–VII) also indicate that the degree of  $\text{Cl}^-$  selectivity produced in these membranes by amphotericin B was affected by the amino and carboxyl groups on that molecule in a manner consistent with the behavior of an amphoteric compound. Since the  $\text{pK}$  of many carboxylic acids is in the range of 4–5 (38), the relatively high values of  $t_{\text{Na}}$  for amphotericin B at  $\text{pH}$  6.0, with reference to the methyl ester of *N*-acetylamphotericin B (Tables I, IV, and VII), may have been due to fixed negative charges on the dissociated carboxyl groups of amphotericin B. In support of this hypothesis, a reduction in  $\text{pH}$  to 2.5, which should titrate the carboxyl group to the undissociated form (39), raised and reduced, respectively, the values of  $t_{\text{Cl}}$  and  $t_{\text{Na}}$  for amphotericin B to those observed with the methyl ester of *N*-acetylamphotericin B (Table VII). Similarly, the lower values of  $t_{\text{Na}}$  for amphotericin B, in comparison to *N*-acetylamphotericin B, at  $\text{pH}$  6.0 but not at  $\text{pH}$  10.55 (Tables IV and VII) imply that at the lower  $\text{pH}$ , positive charges on the amino group of amphotericin B contributed to the degree of  $\text{Cl}^-$  selectivity produced by that compound.

Finally, it should be noted that the mechanism by which amphotericin B and related compounds increase the ionic permeability properties of thin lipid membranes is not understood. The high electrical resistance of unmodified thin lipid membranes may be referable to the properties of the surface monolayers at the interfaces between aqueous and membrane phases (1, 3, 40). Accordingly, it is reasonable to suppose that the polyene-sterol units involved in pore formation modify the properties of these surface monolayers. However, it is not clear whether the polyene-dependent increases in ionic conductance are referable to an increase in the concentration (i.e., equilibrium distribution) and/or the mobility of ionic species within the membrane phase, and presumably, in the aqueous channels. Hopefully, studies currently in progress may provide additional information concerning this issue.

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#### REFERENCES

1. ANDREOLI, T. E., J. A. BANGHAM, and D. C. TOSTESON. 1967. The formation and properties of thin lipid membranes from HK and LK sheep red cell lipids. *J. Gen. Physiol.* 50:1729.



2. ANDREOLI, T. E., V. W. DENNIS, and A. M. WEIGL. 1969. The effect of amphotericin B on the water and nonelectrolyte permeability of thin lipid membranes. *J. Gen. Physiol.* **53**:133.
3. ANDREOLI, T. E., and M. MONAHAN. 1968. The interaction of polyene antibiotics with thin lipid membranes. *J. Gen. Physiol.* **52**:300.
4. DENNIS, V. W., M. A. KELEMAN, and T. E. ANDREOLI. 1968. Amphotericin B-dependent pore formation in thin lipid membranes. Abstracts of the American Society of Nephrology 2nd Annual Meeting. Washington, D. C. 13.
5. STEAD, N. W., V. W. DENNIS, and T. E. ANDREOLI. 1969. Polyene-dependent pores in thin lipid membranes. Abstracts of the Biophysical Society 13th Annual Meeting. Los Angeles, Calif. A-35.
6. MUELLER, P., D. O. RUDIN, H. TI TIEN, and W. C. WESCOTT. 1962. Reconstitution of excitable cell membrane structure in vitro. *Circulation.* **26**:1167.
7. TIEN, H. T., S. CARBONE, and E. A. DAWIDOWICZ. 1966. Formation of "black" lipid membranes by oxidation products of cholesterol. *Nature (London).* **212**:718.
8. TIEN, H. T. 1967. Black lipid membranes in aqueous media: interfacial free energy. *J. Phys. Chem.* **71**:3395.
9. STAVERMAN, H. J. 1951. The theory of measurement of osmotic pressure. *Rec. Trav. Chim. Pays-Bas.* **70**:344.
10. ANDREOLI, T. E., M. TIEFFENBERG, and D. C. TOSTESON. 1967. The effect of valinomycin on the ionic permeability of thin lipid membranes. *J. Gen. Physiol.* **50**:2527.
11. FINKELSTEIN, A., and A. CASS. 1968. Permeability and electrical properties of thin lipid membranes. *J. Gen. Physiol.* **52** (1, Pt. 2):145.
12. VAN ZUTPHEN, H., L. L. M. VAN DEENEN, and S. C. KINSKY. 1969. The action of polyene antibiotics on bilayer lipid membranes. *Biochem. Biophys. Res. Commun.* **22**:393.
13. FINKELSTEIN, A., and A. CASS. 1967. Effect of cholesterol on the water permeability of thin lipid membranes. *Nature (London).* **216**:717.
14. DAINTY, J. 1963. Water relations of plant cells. *Advan. Bot. Res.* **1**:279.
15. DAINTY, J., and C. R. HOUSE. 1966. "Unstirred layers" in frog skin. *J. Physiol. (London).* **182**:66.
16. CASS, A., and A. FINKELSTEIN. 1967. Water permeability of thin lipid membranes. *J. Gen. Physiol.* **50**:1765.
17. EVERITT, C. T., W. R. REDWOOD, and D. A. HAYDON. 1969. The problem of boundary layers in the exchange diffusion of water across biomolecular lipid membranes. *J. Theor. Biol.* **22**:20.
18. DEMEL, R. A., F. J. L. CROMBAG, L. L. M. VAN DEENEN, and S. C. KINSKY. 1968. Interaction of polyene antibiotics with single and mixed lipid monomolecular layers. *Biochim. Biophys. Acta.* **150**:1.
19. OROSHNIK, W., and A. D. MEBANE. 1963. The polyene antifungal antibiotics. *Fortschr. Chem. Org. Naturst.* **17**.
20. COPE, A. C., V. AXEN, E. P. BURROWS, and J. WEINLICH. 1966. Amphotericin B. I. Carbon skeleton ring size and partial structure. *J. Amer. Chem. Soc.* **88**:4228.
21. CEDER, O., and R. RYHAGE. 1964. The structure of filipin. *Acta Chem. Scand.* **18**:558.
22. COPE, A. C., C. P. BURROWS, M. E. ERIEG, S. MOON, and W. WIRTH. 1965. Rimocidin. I. Carbon skeleton, partial structure and absolute configuration at C-27. *J. Amer. Chem. Soc.* **87**:5452.
23. LIPPE, C. 1968. Effect of amphotericin B on thiourea permeability of phospholipid and cholesterol bilayer membranes. *J. Mol. Biol.* **35**:365.
24. LIPPE, C. 1969. Urea and thiourea permeabilities of phospholipid and cholesterol bilayer membranes. *J. Mol. Biol.* **39**:669.
25. TIEN, H. T., and A. L. DIANA. 1967. Black lipid membranes in aqueous media: effect of salt on electrical properties. *J. Colloid Sci.* **24**:287.
26. HANAI, T., D. A. HAYDON, and J. TAYLOR. 1964. An investigation by electrical methods of lecithin-in-hydrogen films in aqueous solutions. *Proc. Roy. Soc. Ser. A.* **281**:377.

27. TIEN, H. T., and A. L. DIANA. 1968. Bimolecular lipid membranes: a review and summary of some recent studies. *Chem. Phys. Lipids*. 2:55.
28. HUANG, C., L. WHEELDON, and T. E. THOMPSON. 1964. The properties of lipid bilayer membranes separating two aqueous phases: formation of a membrane of simple composition. *J. Mol. Biol.* 8:148.
29. MIYAMOTO, V. K., and T. E. THOMPSON. 1967. Some electrical properties of lipid bilayer membranes. *J. Colloid Sci.* 25:16.
30. HANAI, T., D. A. HAYDON, and J. TAYLOR. 1965. The influence of lipid composition and of some absorbed proteins on the capacitance of black hydrocarbon membranes. *J. Theor. Biol.* 9:422.
31. TIEN, H. T., and H. P. PING. 1968. Permeation of water through bilayer lipid membranes. *J. Colloid Sci.* 27:1968.
32. FIESER, L. F., and M. FIESER. 1959. Steroids. Reinhold Publishing Corp., New York. 9.
33. MISLOW, K. 1966. Introduction to Stereochemistry. W. A. Benjamin, Inc., New York. 76.
34. BLADON, P. 1958. Chemistry. In Cholesterol, Chemistry, Biochemistry, and Pathology. R. P. Cook, editor. Academic Press, Inc., New York. 28.
35. ROBERTS, J. D., and M. C. CASERIO. 1965. Basic Principles of Organic Chemistry. W. A. Benjamin, Inc., New York. 1152.
36. FIESER, L. F., and M. FIESER. 1959. Steroids. Reinhold Publishing Corp., New York. 35.
37. TEORELL, T. 1953. Transport processes and electrical phenomena in ionic membranes. *Progr. Biophys. Mol. Biol.* 3:305.
38. ROBINSON, R. A., and R. H. STOKES. 1959. Electrolyte Solutions. Butterworth and Co. (Publishers) Ltd. 2nd edition. 527.
39. HELFFERICH, F. 1962. Ion Exchange. McGraw-Hill Book Co., New York. 82.
40. TOSTESON, D. C., T. E. ANDREOLI, M. TIEFFENBERG, and P. COOK. 1968. The effects of macrocyclic compounds on cation transport in sheep red cells and thin and thick lipid membranes. *J. Gen. Physiol.* 51:(5: Pt. 2):373.